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14. ABSTRACT

The purpose of this award is to test in preclinical models the hypothesis that cytotoxic conjugates of elemental selenium and proteins are safe and effective for the systemic therapy of invasive breast cancer. The grant has three specific aims, 1) to evaluate the safety and efficacy of systemically administered Se(0)-protein conjugates in athymic nude mice bearing xenografts of human breast cancer cells, 2) to assess the functional integrity of conjugate-treated normal human hematopoietic stem cells, and 3) to determine by use of the combination index method how Se(0)-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of invasive breast cancer. We report here on the preparation and evaluation of high-potency cytotoxic Se(0)-protein conjugates, a quantitative in vitro analysis of the interactions of Se(0)-protein conjugates with other chemotherapeutic agents, the surprise finding that certain antibiotics can interfere with the generation of cytotoxic conjugates, and a simple and rapid spectroscopic method to detect such interferences.

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Introduction

Early detection, adjuvant hormone therapy, and adjuvant chemotherapy have improved survival rates in breast cancer. However, for patients with advanced disease, the prognosis remains poor with 5-year survival rates as low as 23% for patients with distant metastases. In the 1990s, large numbers of breast cancer patients were treated with high-dose chemotherapy and autologous stem cell transplants. The expectation was that the dose escalation afforded by the autologous stem cell transplants would have a major impact on survival. However, controlled trials have failed to show a significant advantage of high-dose chemotherapy over standard therapy. It thus appears that currently available forms of chemotherapy - even when used at high doses - are unable to reliably eradicate the disease in patients with high-risk breast cancer.

Most of the currently used anti-cancer drugs were developed based on their good performance in leukemia/lymphoma-based screening systems. Not surprisingly, they tend to perform best when used in the treatment of leukemias and lymphomas. Major breakthroughs in the treatment of breast cancer most likely require new agents whose mechanism of action is different from that of typical anti-leukemia/lymphoma drugs.

Grant W81XWH-04-1-0525 proposes to assess the safety and efficacy of a novel class of cytotoxic agents whose mechanism of action is fundamentally different from that of established anti-cancer drugs. The novel cytotoxic agents consist of high-affinity conjugates of extremely small (subnano) particles of elemental selenium (Se[0]) and (lipo)proteins. The (lipo)protein component acts as a Trojan horse that delivers the cytotoxic entity (selenium in oxidation state zero) to breast cancer cells as part of a physiological process. It exploits the fact that breast cancer cells have an increased requirement for serum albumin (and possibly also lipoproteins) and, therefore, an increased capacity to bind and internalize albumin.

Breast cancer cells internalize Se(0)-protein conjugates by an endocytotic process. Once inside their target cells, Se(0)-protein conjugates act as air oxidation catalysts that rapidly deplete cells of glutathione and induce a loss of mitochondrial potential, a loss of plasma membrane asymmetry, and the activation of several caspases. The cytotoxic action of Se(0)-protein conjugates is not cell-cycle specific and appears to be only minimally affected by drug resistance mechanisms. Se(0)-protein conjugates potentiate - often synergistically - the cytotoxic effects of ionizing radiation and several standard chemotherapeutic agents.

Incorporating Se(0)-protein conjugates into the treatment of invasive breast cancer may prove particularly rewarding because breast cancer tissue is known to accumulate exceptionally large quantities of serum albumin. Typically, about one fifth of the cytosolic protein content of breast cancer cells consists of serum albumin. Despite low blood flow in breast cancer tissue, albumin clearance is very high in breast cancer tissue, and albumin extraction is 3-20 times higher than in any normal tissue. The inverse correlation between albumin content and estrogen-receptor expression suggests that Se(0)-protein conjugates may prove particularly useful in the treatment of estrogen-receptor negative breast cancer.

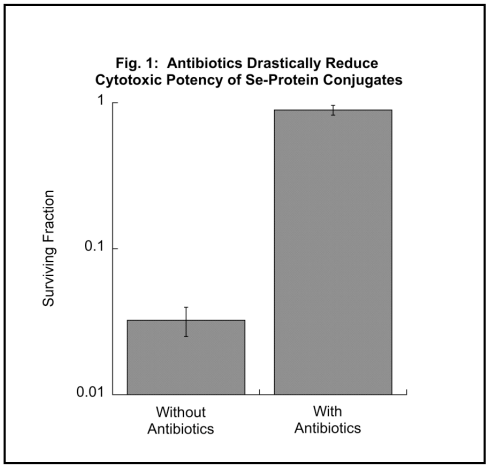
Grant W81XWH-04-1-0525 is designed to test the hypothesis that proteinated subnano particles of elemental selenium can be developed into safe and effective agents for the systemic treatment of invasive breast cancer if used either as single-modality

agents or in combination with certain other drugs. The grant has 3 specific aims. 1) It will evaluate the safety and efficacy of systemically administered Se(0)-protein conjugates in athymic nude mice bearing xenografts of MCF7 or MDA-MB-435 human breast cancer cells. 2) It will assess the *functional* integrity of conjugate-treated normal human hematopoietic stem cells. 3) It will determine by use of the combination index method how Se(0)-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of invasive breast cancer. The objective is to identify drug combinations and dose schedules that are synergistic or at least additive with regard to the depletion of breast cancer cells but well tolerated by normal cells.

Body

Task 1: Preparation and In Vivo Evaluation of High-Potency Cytotoxic Se(0)-protein Conjugates.

An in vitro evaluation of cytotoxic Se(0)-protein conjugates under high serum conditions has shown that is difficult to prepare sufficiently potent Se(0)-protein conjugates using the selone dye MC54 as a starting material. The main problem is that when dye concentrations reach about 80 μM, the photobleaching process slows down sharply. We attribute the problem to the lipophilic nature of selenomerocyanine dyes with expanded back rings and their tendency to form aggregates in aqueous media. We therefore have placed orders for the custom synthesis of two analogues of MC54 that have less lipophilic benzoxazole or benzthiazole back rings instead of the naphth[2,1-d]thiazole back ring of MC54. Both analogues are significantly less lipophilic and are therefore less prone to aggregate formation, which should facilitate the preparation of high-potency conjugates. The analogue with the benzthiazole back ring will also generate green-fluorescent photoproduct-albumin conjugates like MC54, which may be useful for drug localization and drug uptake studies. The analogue with the benzoxazole back ring will not generate green-fluorescent photoproduct-albumin conjugates but may further reduce aggregate formation and, thereby, facilitate the production of high-potency conjugates.



Both structural analogues have been synthesized previously on a small scale. We do not anticipate encountering major technical difficulties during the scale-up.

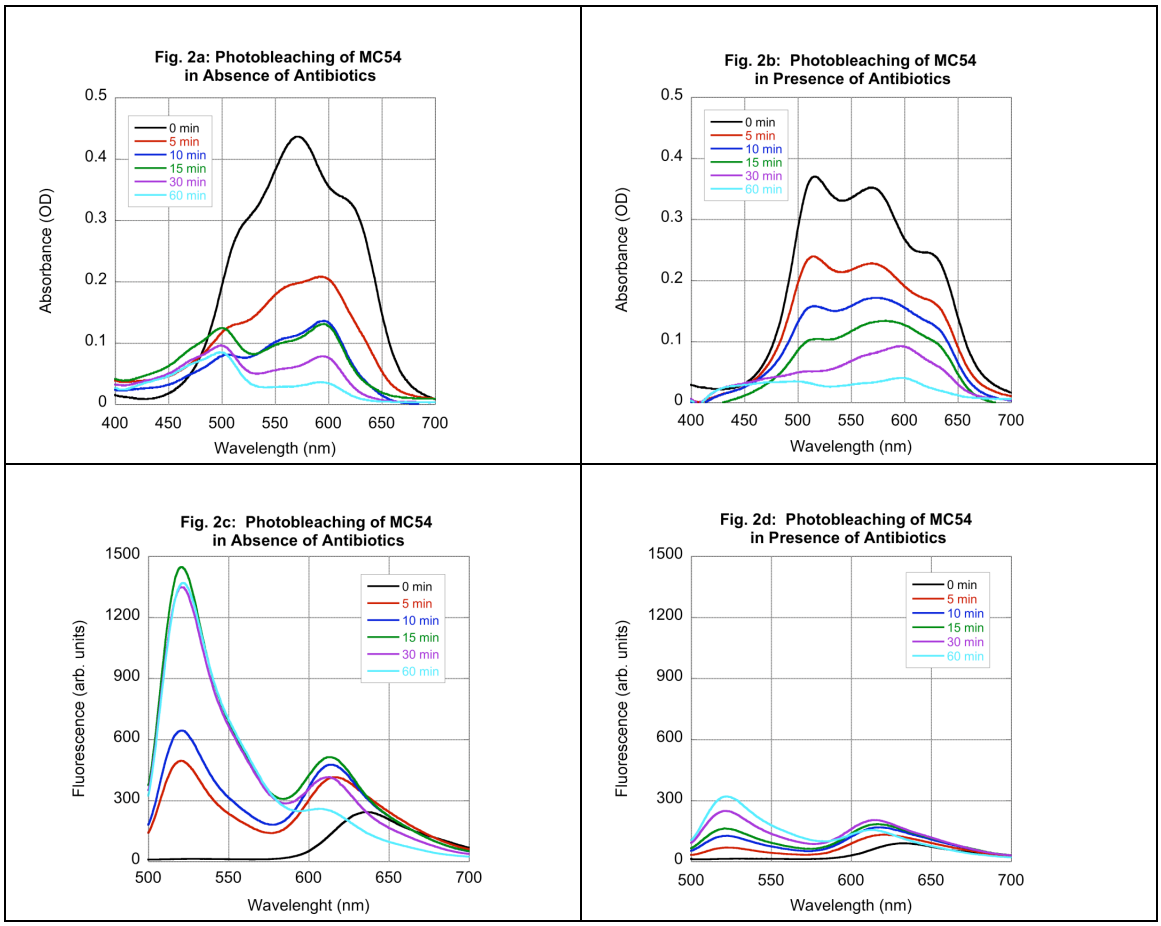
As an inexpensive alternative to the photobleaching of selone dyes, we have explored the feasibility of generating cytotoxic Se(0)-protein conjugated by the chemical reduction of selenium dioxide with ascorbic acid in the presence of serum albumin. We obtained preparations with significant cytotoxic activity. However, cytotoxic potencies were almost two orders of magnitude lower than those achieved by the photobleaching of selone dyes. Extrapolations of dose-response curves suggest that it is impossible to match the small size and cytotoxic potency of photochemically

generated Se(0)-protein conjugates without resorting to extremely high albumin concentrations. Therefore, our photochemical method remains the method of choice for generating high-potency cytotoxic Se(0)-protein conjugates.

Interference of Antibiotics With Generation of Cytotoxic Se(0)-protein Conjugates and Green-Fluorescent Photoproduct-Albumin Conjugates

During this past year, a leak in a RO water storage tank caused extensive flooding in our laboratory area. After the flooding incidence, we had major problems with infections in our cell cultures, which eventually forced us to add antibiotics (penicillin/streptomycin) to our culture media. Shortly after the flooding incidence, a failure in the regulatory circuit of the air conditioning system maintained room temperatures in the laboratory at 113 °F for an extended period of time. This caused multiple equipment failures and a loss of all cell cultures. When we reestablished the lost cultures from frozen stock, the tumor cell lines appeared to have become almost completely resistant to cytotoxic conjugates (Fig. 1). The finding was confirmed with multiple cell lines from authentic stock, ruling out an accidental mix-up of cell lines. We initially suspected heat damage to equipment and/or reagents (e.g. the starting material used to generate cytotoxic conjugates) as the cause of the problem but eventually realized that antibiotics had been added not only to the culture medium used for cell maintenance and clonal assay but also to the medium used for the production of cytotoxic conjugates. As we found out, the presence of antibiotics greatly reduced the cytotoxic potency of photobleached MC54 (Fig. 1), at least in part by causing extensive dye aggregation. As Fig. 2a and Fig. 2b show, in the presence of antibiotics, the absorption spectrum of MC54 showed a reduced concentration of photochemically active monomers (absorbing at about 625 nm) and an enhanced concentration of dimers and higher aggregates characterized by their absorption maxima at shorter wavelengths. The production of green-fluorescent photoproduct-albumin conjugates was reduced almost 5-fold in the presence of antibiotics (Fig. 2c and 2d). The reduction of the fluorescence emission peak at 520 nm was not due to an artifact such as fluorescence quenching, as the corresponding absorption peak was reduced proportionally. A similar suppression of fluorescent conjugate formation was achieved using the drug flufenamic acid, suggesting that the chromophore photoproduct involved in the formation of green-fluorescent photoproduct-albumin conjugates needs access to the Type II binding site of albumin.

Studies on the interference of antibiotics with conjugate formation were not part of the original research plan. However, we felt compelled to investigate the problem for two reasons, 1) because being able to generate cytotoxic conjugates of predictable potency was a prerequisite for all three specific aims of the grant, and 2) because of its obvious relevance for future clinical applications of Se(0)-protein conjugates. Cancer patients are often neutropenic and, therefore, at increased risk for infections. Prophylaxis with antibiotics reduces the risk of infections, but, as this study shows, may interfere



with the cytotoxic activity of this experimental anti-cancer drug. Since we consider using the patient's own serum for the preparation of cytotoxic conjugates, understanding the interactions of antibiotics with conjugates is essential. We do not want to generalize based on the experience with only two antibiotics. However, we want to point out that the potential for interference exists and that the issue needs to be addressed when using patient-derived serum proteins as carrier proteins. Fig. 2 suggests that a spectroscopic analysis may provide a quick and simple way to detect problematic interactions.

Task 3: Interactions of Cytotoxic Se(0)-protein Conjugates With Other Chemotherapeutic Agents

Work on Task 3 is progressing according to plan. With the exception of amifostine, all drugs are producing suitable dose-response curves in the standardized cytotoxicity assay. This means, that we can accomplish at least a 1-log reduction of in vitro clonogenic cells by a short-term (1-hour) incubation with a single drug. Amifostine's failure to kill breast cancer cells was not unexpected. With the exception of some leukemia cell lines, amifostine appears to be non-toxic to most tumor cells. However, amifostine sometimes significantly enhances the anti-cancer activity of other drugs while protecting normal cells. This is the main reason why amifostine will remain included it in the combination drug protocols that are evaluated in Task 3.

Key Research Accomplishments

- The in vitro analysis of cytotoxic Se(0)-protein conjugates under high-serum conditions has shown that it is difficult to produce conjugates with sufficient potency using the selenomerocyanine dye MC54 as a starting material. We attribute the problem to the limited solubility of MC54 in water and the pronounced tendency of MC54 to form aggregates. We hope to overcome the difficulty by switching to less lipophilic analogues of MC54.
- We made the unexpected observation that antibiotics (penicillin/streptomycin) interfere with the generation of cytotoxic and fluorescent conjugates if they are present during the photobleaching of the selone dye. The finding has important practical implications for future clinical applications of Se(0)-protein conjugates.
- The quantitative evaluation of cytotoxic Se(0)-protein conjugate in conjunction with other chemotherapeutic agents is progressing according to plan with all but one drug (amifostine) generating useful dose-response curves in the standard cytotoxicity assay.

Reportable Outcomes

***New Funding**:*

I have applied for and received a pilot grant entitled "Nano-Selenium for the Mitigation of Radiation Injuries" (U19-AI067734) from the Center for Medical Countermeasures Against Radiological Terrorism (CMCRT). The application benefited substantially from the research that is supported by W81XWH-04-1-0525. While primary task of the CMCRT is to develop drugs that mitigate radiation injuries sustained during an act of terrorism or a nuclear accident, all work performed under this grant also has implications for the mitigation and treatment of radiation injuries in cancer patients who receive high-dose ionizing radiation as part of their therapeutic regimen. The CMCRT grant focuses on elemental selenium particles that are larger than the ones used for the breast cancer project and have anti-oxidant rather than pro-oxidant properties. Since the CMCRT grant will specifically address the size issue, we hope to learn in which size range the critical transition from anti-oxidant to pro-oxidant/cytotoxic characteristics happens and if the inexpensive method used to generate nano-Se with anti-oxidant properties can eventually be adapted to generate species with pro-oxidant/cytotoxic properties.

Conclusions

The limited solubility of the pro-drug MC54 makes the production of high-potency Se(0)-protein impractical. We therefore have initiated the custom synthesis of two structural analogues that are less lipophilic.

The in vitro evaluation of the safety and efficacy of Se(0)-protein conjugates used in combination with other chemotherapeutic agents is progressing according to plan.

We have made the unexpected observation that therapeutic concentrations of antibiotics (penicillin/streptomycin) can interfere with the production of cytotoxic Se(0)-protein conjugates and green-fluorescent photoproduct-albumin conjugates. The observation has practical implications for future clinical applications of cytotoxic Se(0)-protein conjugates. The spectrophotometric analysis that was used to diagnose the problem may provide a simple and rapid method to screen antibiotics for potential interference with cytotoxic conjugate formation.

References

None

Appendix

None